In Vivo Fate of Monoclonal Antibody B72.3 in Patients with Colorectal Cancer

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Radiolabeled B72.3 (anti-TAG-72) has been shown to selectively localize metastatic lesions in 70%–80% of the cases. Serum samples from 27 colorectal carcinoma patients who received iodine-131- (^{131}I) B72.3 by i.v. administration were analyzed. Circulating immunoreactive antibody followed a biphasic clearance pattern. High-performance liquid chromatography (HPLC) and SDS-polyacrylamide gel electrophoresis demonstrated that ^{131}I-B72.3 retained its integrity in the patients' sera. HPLC analysis also demonstrated the presence of immune complexes in the sera of 12 patients; this correlated with elevated levels of circulating TAG-72. Several different HAMA response patterns were detected in the 25 patients' sera that were analyzed; some patients developed HAMA as early as 5–7 days post-MAb injection. Higher doses of administered MAb B72.3 correlated with the development of HAMA ($p = 0.007$). The presence of elevated levels of TAG-72 in the patients' pre-inoculum serum was shown to correlate with the detection of lesions by gamma scanning. Serum TAG-72 may serve as a criteria for patient selection for immunodiagnostic or immunotherapeutic procedures using MAb B72.3.

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Radiolabeled monoclonal antibodies (MAbs) reactive with tumor-associated antigens have been effectively utilized in patients for the detection of carcinoma by gamma camera imaging (*1–7*). The optimal targeting of tumor lesions by MAbs for diagnostic or therapeutic applications requires that the MAb retain its integrity and immunoreactivity following administration in patients. To date, the majority of clinical, as well as preclinical, studies have primarily involved radioimaging following administration of radiolabeled MAb (*1–7*) or examination of therapeutic efficacy of MAbs either alone (*8–10*) or conjugated with drugs (*11,12*), toxins (*13,14*), or radionuclides (*15–18*). Several investigators have presented pharmacokinetic studies of various MAbs (*3,19–24*). These reports have shown that the circulating half-lives for individual antibodies vary and are dependent upon tissue reactivities and the dose of MAb administered. The plasma half-life of one MAb (17-1A), administered intravenously to colorectal carcinoma patients, varies from 1 day to 20 days for single doses ranging from 15 mg to 440 mg (*25*). Doses ranging from 0.2 to 20 mg of iodine-131- (^{131}I) B72.3 have resulted in a circulating $T_{1/2}$ of ~3 days (*21*).

The immunoglobulin pharmacokinetics can be influenced by a number of other factors. These include the integrity and immunoreactivity of the MAb, the presence of circulating antigen, and the presence of anti-mouse immunoglobulin antibodies (HAMA). The presence of circulating antigen or HAMA could potentially result in the formation of immune complexes, either antigen-antibody or antibody-antibody complexes, and subsequently prevent effective targeting by binding with the MAb. Formation of immune complexes may also lead to altered pharmacokinetics (*26,27*) and could result in complications such as immune complex disease or anaphylactoid reactions. Due to this potential, it is important to analyze patients' sera for the formation of immune complexes, and, to assess the effect of antigen-antibody or antibody-antibody complexes on the pharmacokinetics and localization of administered MAb. The few studies that have addressed the in vivo fate of MAbs are limited and only cover a 1-2-day period. In those studies administering radiolabeled MAb to patients, the pharmacokinetics of the MAb have been determined by following the radioactivity associated with the MAb (*18–19,21,24*). Effects of circulating antigen on in vivo localization of administered antibody have been reported for 19-9 (*3*) and OC-125 (*24,28*).

MAb B72.3, an IgG_1, (*30–32*), binds to a high molecular weight glycoprotein, termed TAG-72, which has been shown to have properties of a mucin (*33*). Using immunohistochemical, immunocytochemical, and radioimmunoassay techniques, MAb B72.3 has been shown to react with over 90% of colorectal, gastric, and ovarian carcinomas and 70% of breast carcinomas (*31*,

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32,34). In previous clinical studies, radiolabeled B72.3, administered intravenously or intraperitoneally, has been shown to localize primary and metastatic lesions of colorectal and ovarian carcinoma patients (*5–6, 35–36*). Intravenous (i.v.) administration to colorectal carcinoma patients of ^{131}I-B72.3 IgG has resulted in localization of the MAb in ~75% of the metastatic lesions found at various body sites (*5,21,35*) with ~50% of the patients being positive by radioimmunoscintigraphy. Paired-label studies have demonstrated the specificity of B72.3 localization following i.v. or intraperitoneal (i.p.) administration (*5,6*). Studies using an intraoperative hand-held gamma detecting probe have demonstrated the localization of ^{125}I-B72.3 in 83% of primary and recurrent colorectal cancer lesions in 37 patients (*36*).

In this study, we have analyzed the sera from colorectal carcinoma patients who have received ^{131}I-B72.3 IgG by i.v. administration. The serum samples were analyzed for the presence of TAG-72, the amount of circulating immunoreactive MAb B72.3 IgG, the amount of radionuclide associated with the MAb, and the integrity of the radiolabeled B72.3 IgG. The presence and formation of HAMA in the serum and the formation of immune complexes also were investigated.

MATERIALS AND METHODS

Patients

Patients with colorectal carcinomas were selected from National Cancer Institute Surgery Branch protocols as previously detailed (*5*). The protocol was approved by the Institutional Human Research Committee of the National Cancer Institute, and all patients gave their informed consent. All but two of the patients had metastatic carcinoma whose primary lesion had been previously resected. The patients' ages ranged from 16 to 70 yr (17 males, 10 females). Many of the patients with recurrent and/or metastatic tumor had advanced disease. Histologic examination revealed that the tumors ranged from well to poorly differentiated adenocarcinomas and included mucinous adenocarcinomas and signet ring cell variants.

The patients received ^{131}I-labeled B72.3 IgG (i.v.) according to different dosing schedules as detailed below. Patients (24 of 27) underwent surgical exploration and resection of tumor 6 to 8 days postinjection of the radiolabeled antibody. Results of the imaging and surgical studies have been previously described (*5,35*).

Antibody Administration and Sample Collection

The generation, characterization, reactivities, as well as the purification of MAb B72.3 have been detailed previously (*30–32*). The purified IgG was filtered and all end lots were tested for the presence of *Mycoplasma*, 12 adventitious viruses and pyrogens and were negative. End lots were also tested for sterility and general safety (*5,6,21*).

B72.3 was labeled with Na^{131}I using the Iodo-Gen method (*37*). Purified IgG (500 μg) was labeled with ~5 mCi of Na^{131}I. The ^{131}I-B72.3 was diluted, vialed, and tested for pyrogenicity and sterility as described elsewhere (*5,6,21*). The immunoreactivity of the radiolabeled antibody was tested in a solid-phase radioimmunoassay (RIA) (*38*).

Patients were administered 0.16 to 20 mg of MAb with 2–10 mCi of ^{131}I, by slow i.v. infusion (over 1 hr). None of the patients demonstrated an adverse reaction during or following MAb infusion. Blood samples were collected 5, 30, 60, 120, and 240 min after MAb administration and at daily intervals until surgery and intermittently following surgery. The sera samples were analyzed for integrity of the radiolabeled antibody by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. The sera samples were also analyzed for the presence of TAG-72, circulating immunoreactive MAb, immune complexes, and for the presence and development of HAMA.

High-Performance Liquid Chromatography

The integrity of the radiolabeled antibody, prior to administration and in the patients' sera, was examined using high-performance liquid chromatography (HPLC). The analyses were performed using Spherogel-TSK 2000 SW (0.75 × 30 cm; Beckman Instruments, Inc., Berkeley, CA) and Spherogel-TSK 3000 SW (0.75 × 30 cm) columns in tandem, equilibrated in 67 m*M* sodium phosphate containing 100 m*M* KCl, pH 6.8. Serum samples (25 μl) were applied and the columns were run at a flow rate of 0.5 ml/min. The protein was detected by absorbance at 280 nm; fractions were collected at 0.5-min intervals and the radioactivity was measured in a gamma scintillation counter.

SDS-Polyacrylamide Gel Electrophoresis

Patient serum samples containing radiolabeled B72.3 were analyzed using discontinuous SDS-PAGE according to the method of Laemmli (*39*). Samples were analyzed under non-reducing conditions on a gradient gel of 3%–15% (w/v) acrylamide (14 × 12.5 × 0.15 cm) and reducing conditions (0.5% β-mercaptoethanol, 3 min at 100°C) using a gradient gel of 5–20% acrylamide. A stacking gel of 3% acrylamide was used for both gradient gels. The radiolabeled antibody was visualized by autoradiography using Kodak (Rochester, NY) XAR x-ray film and DuPont (Wilmington, DE) Lightning-Plus intensifying screens. Films were exposed at −70°C for 1–7 days.

Radioimmunoassay for TAG-72

The patients' pre-study sera were assayed for levels of TAG-72 using a solid-phase sandwich RIA (CA 72-4 RIA, Centocor, Malvern, PA) (*40*). TAG-72 levels of the serum samples were calculated from a standard curve and expressed as Units/ml.

Determination of Immunoreactive MAb B72.3 Levels in Patient Sera

Retention of B72.3 immunoreactivity in serum was tested using a solid-phase RIA after a sufficient time was allowed to elapse for the decay of the ^{131}I to background. Twenty micrograms [in 50 μl of phosphate-buffered saline (PBS)] of an extract from a human breast tumor metastasis (*41*) containing the TAG-72 antigen was added to each well of 96-well polyvinylchloride (PVC) microtiter plates and allowed to dry overnight at 37°C in an unhumidified incubator. The plates were then treated with 100 μl of 5% bovine serum albumin (BSA) in PBS for 1 hr at 37°C to minimize nonspecific absorption. Serial dilutions of the patients's sera were added

in 50 μl of 1% BSA in PBS. Following an overnight incubation at 4°C, plates were washed with 1% BSA in PBS and ^{125}I-labeled goat anti-mouse IgG (75,000 cpm per 25 μl) was added to each well. The plates were incubated for 1 hr at 37°C, washed and the bound radioactivity detected by counting the individual wells in a gamma scintillation counter. The amount of immunoreactive antibody in the serum samples was quantitated by comparison to a B72.3 IgG standard diluted in normal human serum.

The Detection of Anti-Mouse Immunoglobulin Antibody

All sera were screened for anti-mouse immunoglobulin antibody (HAMA) using a sandwich solid-phase RIA. One hundred nanograms of a murine MAb IgG_1 (B6.2) (*30*) was coated on each well of a PVC microtiter plate and then treated with 5% BSA in PBS as described above. The BSA was aspirated and the wells washed once with 0.5 *M* NaCl in 20 m*M* sodium phosphate (pH 7.2) with 0.02% NaN_3 (wash buffer). Patients' sera were serially diluted in 1% BSA in PBS and added to the plates in duplicate (50 μl/well). Following a 20-24-hr incubation at 4°C, the wells were rinsed with wash buffer and ^{125}I-B6.2 IgG in 1% BSA in PBS (100,000 cpm/50 μl, 1.5-5 ng of B6.2 IgG) was added to each well. The plates were incubated for an additional 20–24 hr at 4°C and then rinsed with wash buffer. The bound ^{125}I-B6.2 was detected by cutting individual wells and measuring the radioactivity in a gamma scintillation counter. A positive response was considered to be greater than 2–3 times the level of the control normal human serum. Serum positive for HAMA was used as a control and used as a standard to normalize the interassay variability of the HAMA RIA.

RESULTS

Colorectal carcinoma patients participating in this study were part of National Cancer Institute Surgery Branch protocols that examined the efficacy of chemotherapy following surgical removal of the bulk tumor. Approximately 7 days prior to surgery, patients were administered ^{131}I-labeled B72.3 IgG. Serum samples from each patient were obtained prior to and periodically post-MAb administration.

MAb B72.3 IgG in Patients' Sera

Sera samples of patients injected intravenously with ^{131}I-labeled B72.3 IgG were assessed using a solid-phase RIA, SDS-PAGE, and HPLC techniques to determine: (a) the ability of the circulating MAb to bind to its specific antigen (TAG-72), and; (b) the integrity of the circulating radiolabeled MAb. The amount of immunoreactive MAb B72.3 present in patients' serum samples was quantitated using a solid-phase RIA, as described in Materials and Methods.

Circulating immunoreactive B72.3 was measured in 19 of the 27 patients. In those patients receiving <1 mg of MAb, immunoreactive B72.3 was not reliably detectable after 24 hr. The titration of B72.3 (Fig. 1) in serum samples from two patients, who received similar amounts of MAb (19.24 and 19.7 mg), serves to demonstrate the similarities among patients in the pharmacokinetics of B72.3. Serial dilutions of serum samples from Patient PH (Fig. 1A) exhibit a gradual decrease in the B72.3 titer over the 72 hr period examined. Sera from Patient CC (Fig. 1B) also exhibits a decrease in the level of immunoreactive B72.3 over time. The apparent differences in B72.3 titer between patients PH and CC may be due, in part, to differences in the plasma volumes of the two patients. When the values are normalized to the plasma level observed 5 min post-MAb administration, the pharmacokinetics of the two patients are similar (see below).

Figure 2 illustrates serum clearance of immunoreactive MAb B72.3 from four patients (CC, PH, HF, and TR) who received the highest doses (17.8–19.7 mg) of MAb B72.3. The values are normalized to each patient's serum drawn at 5 min postinjection. The amount of antibody found in the sera follows a biphasic pattern of clearance. There is an initial equilibration phase with a rapid removal of antibody from the sera within the first

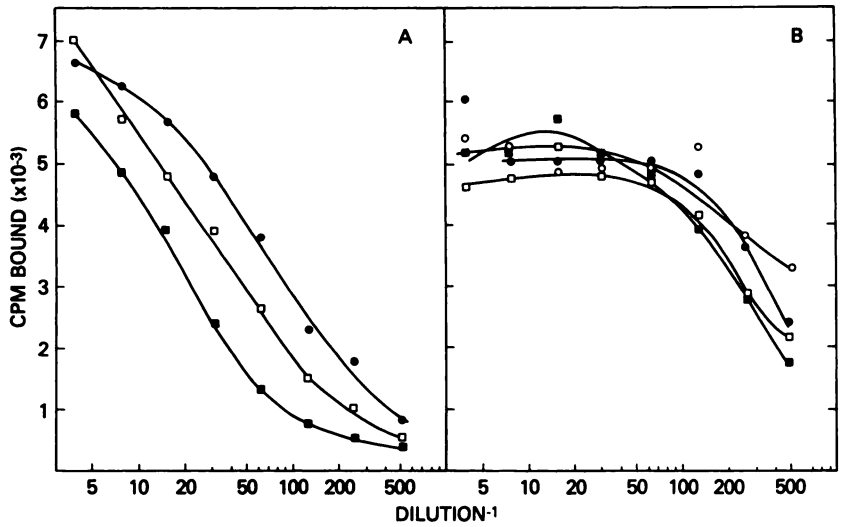

FIGURE 1
Detection of immunoreactive MAb B72.3 in patient sera. Serial dilutions of serum samples from patient PH (A) were drawn at 40 min (●), 24 hr (□), and 72 hr (■) and from patient CC (B) at 5 min (○), 24 hr (●), 48 hr (□), and 72 hr (■) and were reacted with extracts of a human mammary tumor metastasis to the liver that contained the TAG-72 antigen as described in Materials and Methods to determine the presence and quantity of immunoreactive B72.3. Patients PH and CC received 19.7 and 19.24 mg of ^{131}I-B72.3, respectively.

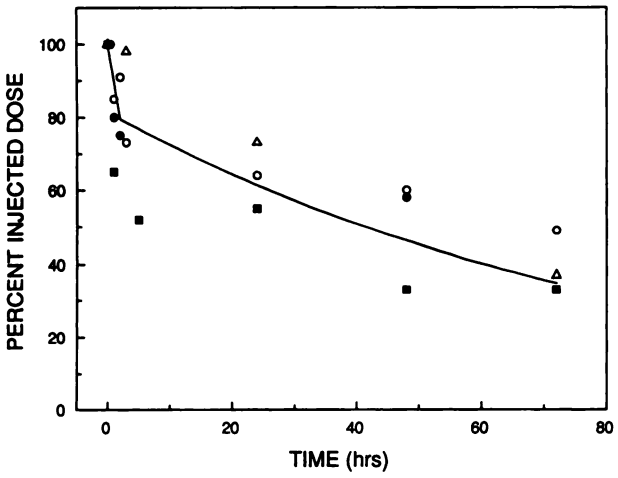

FIGURE 2
Patient serum levels of MAb B72.3 following i.v. administration. Serum levels of immunoreactive MAb B72.3 were detected using a solid-phase RIA described in Materials and Methods; HF (○), TR (●), CC (■), and PH (△). The patients received 19.24, 17.8, 19.24, and 19.7 mg of ^{131}I-B72.3, respectively. The percentage of the injected dose of ^{131}I-B72.3 IgG (solid line) is included for comparison.

4 hr after antibody administration; this is followed by a more gradual clearance of the antibody. While there is a wide range of differences between individual patients, the clearance pattern of the immunoreactive B72.3 follows that of the previously determined ^{131}I-labeled B72.3 (Fig. 2, solid line), suggesting that the radiolabeled B72.3 can be used to follow the serum clearance of the circulating immunoreactive antibody. The pharmacokinetic patterns were similar for the doses of B72.3 ranging from 1 (data not shown) to 20 mg and was independent of whether there was any tumor-specific uptake of the radiolabeled MAb.

Serum samples were subjected to SDS-PAGE analysis with and without disruption by β-mercaptoethanol to examine the integrity of the ^{131}I-labeled B72.3 in patient sera. Samples up to 5 days postinjection were analyzed and the ^{131}I was visualized by autoradiography. Virtually all the radioactivity in the serum is associated with the IgG molecule (Fig. 3A-B). The integrity of ^{131}I-labeled B72.3 was also examined by HPLC analysis at various time points postinjection of the antibody. Serum samples were applied to size-exclusion columns, as described in Materials and Methods, the fractions were collected, and the ^{131}I measured. In all cases, ^{131}I-B72.3 in column buffer and in normal human serum were used as standards. In the majority of the serum samples tested, the radioactivity remains associated with the IgG peak (Fig. 4A).

Analysis of Immune Complexes in Patient Sera

In serum samples from 12 patients, radioactivity was associated with an entity having a higher molecular

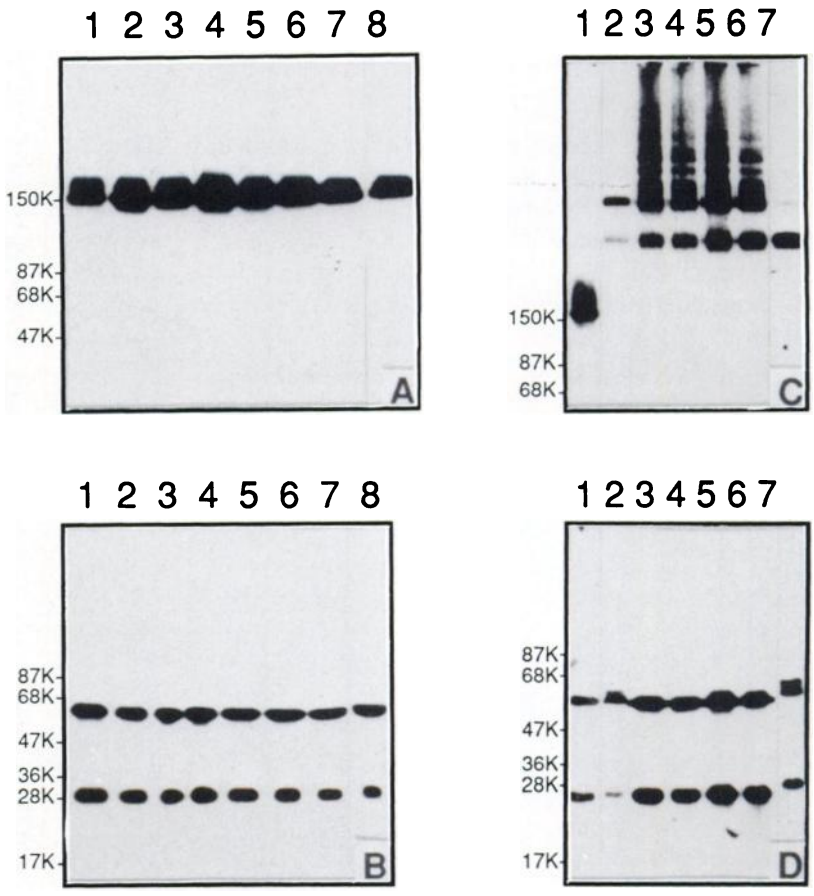

FIGURE 3
SDS-polyacrylamide gel electrophoresis analysis of ^{131}I-labeled B72.3 in patient sera. Serum samples (Patient CC) were analyzed by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. Samples were run under nonreducing conditions (A and C) on a 3%-15% gradient gel and under reducing conditions (B and D) on a 5%-20% gradient gel; Panels A and B, ^{131}I-B72.3 in pre-study serum (Lane 1) and serum samples drawn at 5 min (Lane 2), 1 hr (Lane 3), 2 hr (Lane 4), 3 hr (Lane 5), 24 hr (Lane 6), 48 hr (lane 7) and 72 hr (Lane 8) after MAb administration. Panels C and D, ^{131}I-B72.3 in PBS (Lane 1) and in the pre-study serum (Lane 2). Serum was drawn at 5 min (Lane 3), 30 min (Lane 4), 2 hr (Lane 5), 4 hr (Lane 6), and 24 hr (Lane 7).

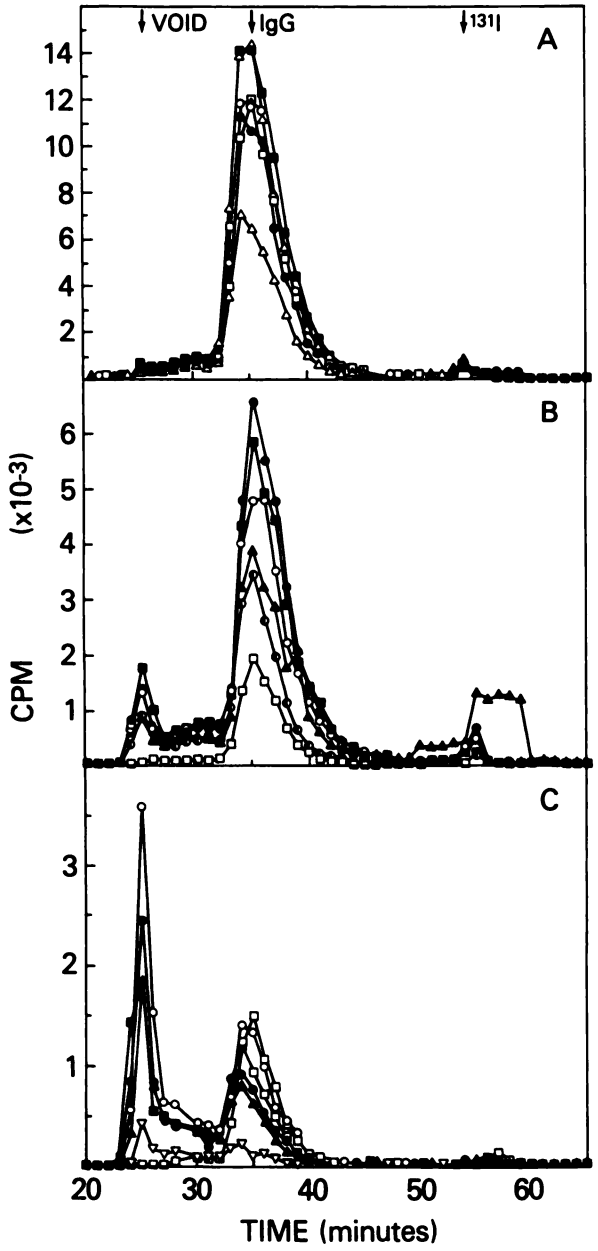

FIGURE 4
HPLC analysis of ^{131}I-B72.3 IgG in patient sera. Serum samples drawn at various time points were applied to a TSK-2000 and TSK-3000 column in tandem arrangement. Fractions were collected and the ^{131}I measured in a gamma scintillation counter. Serum samples from patients BB (A), RC (B), and CP (C) were drawn at 30 min (■), 1 hr (○), 2 hr (●), 4 hr (△), 24 hr (▲), and 96 hr (▽). Iodine-131-B72.3 IgG in 0.2 *M* sodium phosphate, pH 6.8 (□), or in pre-study serum (◐) were used as standards.

weight than IgG which was detected in the column void volume (Fig. 4B). The alteration in the retention time of radiolabeled B72.3 on the columns was observed at the early time points and remained consistent throughout the time points analyzed. In the case of Patient RC (Fig. 4B), an increase in the free ^{131}I was also observed at the 24-hr time point. An additional pattern was observed in several patients in which a large proportion of the radioactivity was detected in the column void volume. In Patient CP (Fig. 4C), ~56% of the ^{131}I was detected in the void volume.

The shift toward the higher molecular entities may be due to either the formation of immune complexes, either antigen-antibody or antibody-antibody, or aggregation of the IgG. The presence of circulating immune complexes was compared to the levels of circulating TAG-72 as measured by the CA 72-4 assay (Table 1). There appears to be a correlation between the formation of immune complexes and the presence of elevated levels (i.e., ≥16 U/ml) of serum TAG-72. The presence of ^{131}I-B72.3 IgG in complexes, however, does not appear to lead to a sequestration of the complexes that results in the alterations of the clearance of the antibody from the plasma. Patients ML, HB, and CP with TAG-72 levels of 16, 67, and 838 U/ml, respectively, whose sera had 11.5%, 24% and 56% of the radiolabeled B72.3 in complexes, all cleared 70%–80% of the ^{131}I-labeled B72.3 by 96 hr (Fig. 5). This is similar to the average clearance of the MAb from the blood of all the patients studied (Fig. 5, solid line).

The formation of immune complexes was also visualized by autoradiography of the SDS-polyacrylamide gels of serum samples from Patient HL. Under nonreducing conditions (Fig. 3C), there is a significant decrease in the migration of the ^{131}I-labeled B72.3. The 30-min, 2-hr, and 4-hr sera exhibit a wide range of indistinct bands, some of which barely entered the 3%–15% gradient gel. By 24 hr, the broad range of bands is no longer apparent and a single, higher molecular weight entity is observed. Formation of immune complexes is very rapid, as evidenced by the formation of a high molecular weight entity when ^{131}I-B72.3 is added to the pre-study sera just prior to loading of the poly-

TABLE 1
HPLC Analysis of Immune Complexes in Patient Serum versus Circulating Antigen Levels

Patient	Serum % MAb in immune complexes	TAG-72 (U/ml)
CP	56.0*	838
EP	9.5	211
RC	6.8	78
HB	24.0	67
MS2	8.5	57
MP	8.0	33
DF	7.0	19
ML	11.5	16
CC	0	2
BB	0	2
CM	0	3

* The percentage of ^{131}I-B72.3 in immune complexes was determined by dividing the cpm associated with the void volume by the total cpm detected times 100.

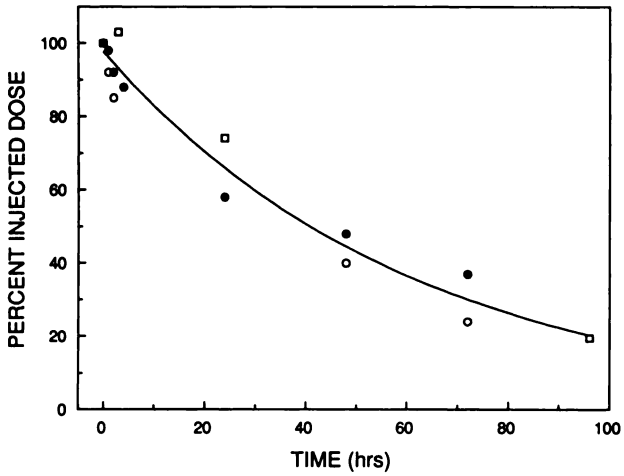

FIGURE 5
Plasma clearance of ^{131}I-labeled MAb B72.3. Plasma clearance was determined by gamma scintillation counting of plasma samples obtained at various times post-injection and normalized for the patients' estimated plasma volume. Values are given for patient ML (●), HB (○), and CP (□) along with the average percent injected dose of the patients (solid line).

acrylamide gel (Fig. 3C; Lane 2). Disruption by β-mercaptoethanol (Fig. 3D) reveals that the antibody retains its integrity, both the heavy and light chain are evident and appear intact.

Analysis of TAG-72 Serum Levels

All patients' pre-study sera were tested for TAG-72 antigen levels using the CA 72-4 assay. A correlation (Pearson's correlation coefficient = 0.771) was found between the presence of elevated TAG-72 and the positive detection of lesions by gamma scanning (Table 2). Of 14 patients with positive scans, 13 patients had serum TAG-72 levels ≥14 U/ml. In only two patients with serum TAG-72 levels of over 14 U/ml (16 and 22 U/ml) were the scans negative (*16*).

TABLE 2
Correlation of TAG-72 Serum Levels with Radioimmunodetection of Tumor by Intravenously Administered ^{131}I-B72.3 IgG

TAG-72* (U/ml)	Scan results (percentage of patients)	
	Positive	Negative
>100	100 (4/4)†	0
50–100	100 (4/4)	0
14–50	71 (4/6)	29
<14	8 (1/12)	93

* Levels of TAG-72 were determined in serum samples using the CA 72-4 RIA. The samples were drawn prior to the administration of ^{131}I-B72.3 IgG.

† Number of patients positive per total number of patients.

Development of Anti-mouse IgG Antibody

Twenty-five of the patients were evaluated for the development of anti-mouse IgG antibodies (HAMA) using a solid-phase sandwich RIA as described in Materials and Methods. The antibody titer was calculated by determining the titer obtained at a given number of counts bound and normalizing that value to a standard serum that is positive for HAMA in order to minimize interassay variability. Serial dilutions of serum samples from patient PH clearly demonstrate a significant anti-mouse IgG antibody response (Fig. 6, inset). Throughout the 121-day period of sampling post-MAb injection,

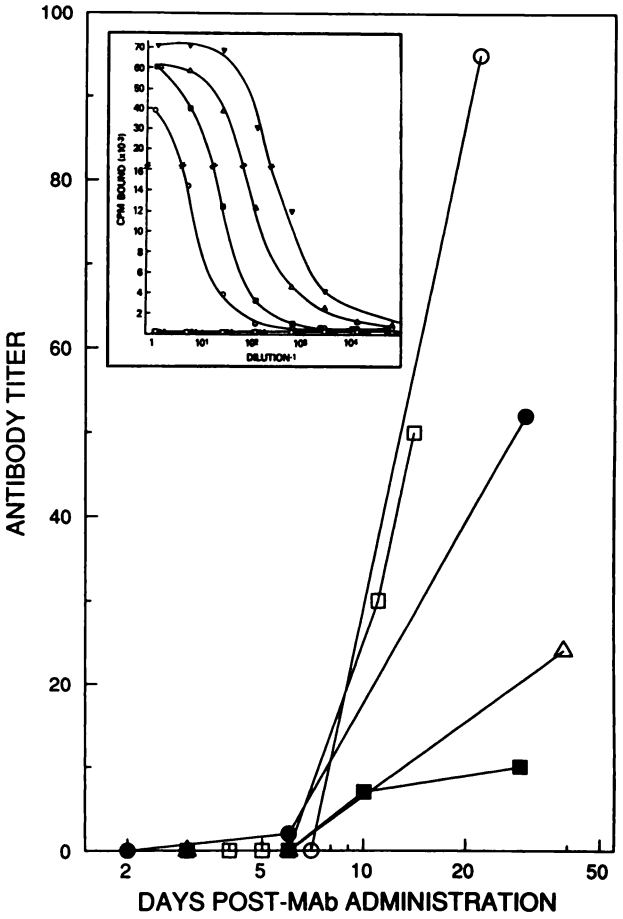

FIGURE 6
Development of anti-mouse IgG antibody in patients following i.v. administration of MAb B72.3. Sera drawn prior to and at various intervals postinjection of MAb B72.3 from patients HF (○), DF (●), BB (□), RS (■), and EL (△) were screened using the sandwich RIA in Materials and Methods. The patients received 19.24, 0.27, 1.36, 1.08, and 3.7 mg, respectively, of ^{131}I-labeled MAb B72.3. The antibody titer was calculated by determining the titer obtained at 2000 cpm and normalizing that value to a control sera positive for HAMA to minimize differences due to interassay variability. Inset: Titration of anti-mouse IgG. Sera from a patient (PH), who received an i.v. dose of MAb B72.3 (19.7 mg), were screened for the presence of HAMA using the sandwich RIA described in Materials and Methods. The sera tested were drawn prior to the study (□); 3 days, (●); 6 days, (▲); 42 days, (○); 58 days, (■); 91 days, (△); and 121 days, (▼) postinjection of MAb B72.3.

the HAMA displays a steady increase in titer. In fact, by 121 days, the HAMA titer is 14,000. It is worth noting that this steady increase in titer is probably not due to the antigen stimulation through the persistence of B72.3 IgG, since 60%–70% of the B72.3 has cleared from the serum by 72 hr (Fig. 2).

Figure 6 illustrates a number of patterns of HAMA responses that have been observed in patients. In general, MAb B72.3 IgG elicits an antibody response in patients that is first detectable at ~5–7 days post-MAb injection. Patient HF presented with an antibody titer of 95 by 20 days, while Patient RS's HAMA response appears to have plateaued after reaching a titer of 10. This pattern may be more consistent with an IgM response and requires further evaluation. Most of the patients that developed HAMA had patterns similar to those of Patients DF, BB and EL, whose antibody titers ranged from 25 to 52. Within the MAb dose ranges specified (Table 3), there appears to be a correlation between HAMA titer and the higher doses of MAb B72.3 administered.

There also appears to be a correlation between the dose of MAb B72.3 and the percentage of patients that are positive for HAMA (Table 3). At the 0.15-0.8-mg dose range, only two (20%) of the patients scored positive for HAMA. The frequency of HAMA development after administration of >0.8 mg of B72.3 IgG is statistically significant as compared to patients who received 0.15-0.8 mg ($p = 0.007$, Student's t-test). In the group of patients that received 0.8-1.5 and 3.7-20 mg, 75% and 71%, respectively, were positive for HAMA. This demonstrates that a dose threshold may exist before a host antibody response is induced by MAb B72.3 IgG. Patients that received doses greater than 3.7 mg generally had higher HAMA titers than patients who received lower doses.

DISCUSSION

Numerous studies have demonstrated the utilization of MAbs for the localization of human malignancies (*1–18*). The pharmacokinetics of several of these murine MAbs have also been reported (*3,19–24*); however, a few studies have focused on the in vivo fate of a monoclonal antibody (*3,24,28,29*). The current study presents a detailed examination of the integrity and immunoreactivity of MAb B72.3 post-administration to colorectal carcinoma patients. In addition, this study presents an analysis of immune complex formation which can be of antibody-antibody or antibody-antigen composition. In the present study, the level of immunoreactive antibody was determined in patient sera using a solid-phase RIA. Comparing the patients who received the highest level of MAb B72.3, when the level of immunoreactive antibody was normalized to the serum drawn at 5 min postinjection, the clearance of the immunoreactive antibody was similar to that of the ^{131}I-B72.3 (Fig. 2). Differences in the serum level of the detectable immunoreactive MAb among patients receiving similar doses (Fig. 1) could be due to: (a) differences in the plasma volumes; (b) the presence of circulating TAG-72 with which MAb B72.3 reacts; or (c) differences in tumor burden. The formation of immune complexes, i.e., the interaction of MAb B72.3 with the circulating antigen, could prevent the detection of B72.3 in patient sera by the solid-phase RIA. The percentage of the B72.3 IgG in circulating immune complexes was found to remain constant in patients as measured by HPLC, therefore a consistent proportion of the immunoreactive MAb would be present in these complexes.

The comparable clearance patterns of the immunoreactive MAb and the ^{131}I-B72.3 suggest that the radionuclide is remaining in association with the MAb in serum following i.v. administration to patients. Similar results have been observed in patients following i.p. administration of ^{131}I-B72.3. This is further supported by the HPLC analysis (Fig. 4) and autoradiography of the SDS-PAGE (Fig. 3). Both reveal that the radioactivity remains in association with the IgG fraction. In most cases, very little free ^{131}I-labeled fragments are detected with the exception of Patient RC (Fig. 4B) where 4%–22% of the radiolabel migrated as free ^{131}I. This does not preclude, however, the possibility that degradation of MAb B72.3 is occurring. Degradation products or free ^{131}I may not be detected due to rapid clearance from the circulation.

Two approaches have been employed for the detection of HAMA. The first implements heavy chain specific (μ and γ) reagents for the detection of the patients' antibody. This approach is restricted by the necessity of diluting the patient serum (1:10 or 1:100) to minimize nonspecific interactions of the serum with the assay components. The second approach, and the one used in this study, has been to use the same MAb as antigen and detecting reagent. This approach has allowed us to test for the presence of HAMA using undiluted sera with minimal background activity allowing the detection of early and low titer (<10) responses.

TABLE 3
Antibody Response of Patients Receiving MAb B72.3

MAb dose (mg)	Total no. of patients	Anti-Mouse IgG Antibody* ++	+	±	−	% Positive†
3.7–20	7	4	1	0	2	71
0.8–1.5	8	0	6	0	2	75
0.15–0.8	10	0	2	2	6	20

* Serum samples, 14–21 days post-administration of MAb B72.3, were assayed for the presence of antibodies to murine IgG as described in Materials and Methods.

† Percentage of patients scored as + or ++.

Patients, e.g. Patient RS (Fig. 6, closed squares), whose titer remained very low, would not have been detected if the sera had to be diluted for the assay. Since many investigators (*42–44*) begin with diluted sera, the development and the frequency of a patient's response to a monoclonal antibody may actually be higher than previously reported. The presence of anti-mouse immunoglobulin antibodies have been associated with diminished MAb localization with subsequent injections of the MAb (*18,29,45*). However, it has also been reported that an immune response does not always prevent the localization of MAb to the tumor (*29,42*).

Detection of HAMA using the sandwich RIA is limited by the presence of circulating tumor-associated antigens found in the sera of a high proportion of patients (*47–50*) and many of these antigens have been shown to be multideterminant. False-positive results would occur if the administered MAb was used in the HAMA RIA. To use a monoclonal antibody in the assay other than the injected MAb would result in not detecting a subset of the anti-mouse immunoglobulin antibody, i.e., anti-idiotypic antibodies. Anti-idiotypic responses have been reported in chronic T-cell leukemia (*43*) and melanoma patients (*44*). Koprowski et al. (*51*) attributed the favorable response of a patient receiving MAb 17-1A to the development of an anti-idiotypic response. Further studies are needed to determine whether the anti-idiotypic response to an injected monoclonal antibody is beneficial or detrimental.

None of the patients in the present study had detectable levels of pre-existing anti-mouse immunoglobulin antibodies. This is in contrast to the report of Schroff et al. (*42*) and Courtenay-Luck et al. (*44*) who have reported that some patients had detectable levels anti-mouse immunoglobulin antibodies before treatment with a MAb. This may be due to: (a) rheumatoid factors (*52*); (b) prior exposure of selected populations to murine tissue; or (c) an artifact of their detection method. Further studies should be performed to evaluate these possibilities.

When evaluating the patients' response to administered MAb B72.3, it was determined that HAMA was first detectable at 5–7 days following injection of the antibody (Fig. 6). An antibody response within one week of MAb injection has also been noted by other investigators (*29*). Several patterns of HAMA response were observed: (a) in some patients, the HAMA reached high titers within a short period of time; (b) other patients display an increase with time, however they do not reach the level seen with the first group; and (c) a patient's response may plateau at a relatively low titer. These different patterns may reflect differences expected from IgG and IgM responses. The response patterns of patients do not seem to correlate with the dose of antibody. There does appear, however, to be a correlation between the dose given to a patient and the frequency of an anti-mouse immunoglobulin response and the titer obtained. A higher frequency of a HAMA response has also been observed in a limited number of patients who have received MAb B72.3 by i.p. injection.

In general, investigators have approached the assessment of the levels of circulating MAb by two methods. When administering a radiolabeled MAb to a patient, immunoreactivity is usually analyzed by incubating the patient sera with the antigen the monoclonal antibody recognizes and measuring the radioactivity bound, however this method assesses the immunoreactivity of only the subset of MAb that carries the radionuclide. The second approach has been to analyze patient sera for total circulating murine IgG (*18–19,22–23*). We have chosen to measure the total level of circulating immunoreactive MAb and compared it to the levels of ^{131}I-B72.3.

Three profile patterns were observed by the HPLC analysis (Fig. 4). In the majority of the patients, the radiolabel eluted with a retention time consistent with that of IgG. In other patients, a small percent of the radioactivity eluted in the void volume, while with others, a large proportion of the radioactivity was associated with the void volume, suggesting the formation of immune complexes or aggregation of the MAb. The formation of this higher molecular weight entity correlates with elevated antigen levels in patient sera, suggesting that antigen-antibody complexes are forming. Other investigators have also observed altered migration of MAbs using column chromatography that was due to either antigen-antibody (*1,24*) or antibody-antibody (*30*) complex formation. Mach et al. (*1*) found that the formation and degree of immune complexes correlated with the level of carcinoembryonic antigen (CEA) detected in the sera of patients. On the other hand, Hnatowich et al. (*24*) have demonstrated immune complex formation with MAb OC125 in the presence of antigen (CA 125), however, it did not correlate with the level of circulating antigen. We have found that immune complex formation occurs rapidly, as evidenced by its presence in the earliest sera drawn and remained constant for a given patient in the sera drawn at all times tested (up to 4 days). These complexes were detected both by HPLC and SDS-PAGE analysis. Interestingly, the presence of the immune complexes did not appear to alter the clearance pattern of the MAb (Fig. 5). Furthermore, the presence of immune complexes does not appear to interfere with the binding of MAb B72.3 to tumor. In similar studies with patients receiving ^{131}I-B72.3 by an i.p. route, circulating immune complexes have also been detected.

It was also of interest that the elevated serum TAG-72 levels correlated with the obtaining of a positive scan (Pearson's correlation coefficient = 0.771). Positive levels of serum TAG-72 (>4.0 U/ml) have been demonstrated in 25%, 35%, 50%, 38%, 29%, and 60% of

patients with primary carcinomas of the esophagus, stomach, colorectum, pancreas, breast and ovary, respectively (*53*). In those with recurrent disease, the percentage of patients with positive serum TAG-72 levels was higher (*53*). Therefore, TAG-72 may be clinically useful as a tumor marker for the monitoring of carcinoma patients. In view of the positive correlation with obtaining positive scan results, the level of serum TAG-72 may also serve as a criteria for the consideration of a patient for either diagnostic or therapeutic studies with MAb B72.3.

This study and others have demonstrated that a significant percentage of patients develop an anti-mouse immunoglobulin response following administration of a murine MAb (*1,8,9,29,42–44*). The problem of immunogenicity may be reduced through the use of fragments of MAbs, recombinant/chimeric MAbs, or human MAbs. A lower immunogenicity would permit multiple injections which in turn would expand the diagnostic usefulness of a monoclonal antibody. It would also permit fractionation of doses for radioimmunotherapy, which may be required for limiting toxicity to normal tissues. Recombinant/chimeric MAbs offer the advantage of tailoring the MAb for a specific application. Constructs with different heavy chain sequences, altered sequences or deleted heavy chain sequences can be generated. In this manner, a MAb might be produced with particular pharmacokinetic characteristics, or, for mediating different effector cell functions.

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